

Halide Binding by the Purified Halorhodopsin Chromoprotein

II. NEW CHLORIDE-BINDING SITES REVEALED BY ^{35}Cl NMR*

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Halorhodopsin is a light-driven chloride pump in the cell membrane of *Halobacterium halobium*. Recently, a polypeptide of apparent $M_r = 20,000$ has been purified that contains the halorhodopsin chromophore. Here we use ^{35}Cl NMR to show that the purified chromoprotein possesses two previously unknown classes of chloride-binding sites. One class exhibits a low affinity ($K_D \gg 1$ M) for chloride and bromide. The second class exhibits a higher affinity ($K_D = 110 \pm 50$ mM) for chloride and also binds other anions according to the affinity series $\text{I}^- > \text{SCN}^- > \text{Br}^- > \text{NO}_3^- > \text{Cl}^- > \text{F}^-$, citrate. Both classes of NMR site remain intact at pH 11, indicating that the essential positive charges are provided by arginine. Also, both classes are unaffected by bleaching, suggesting that the sites are not in the immediate vicinity of the halorhodopsin chromophore. Although the chromoprotein also appears to contain the chloride-transport site (Steiner, M., Oesterhelt, D., Ariki, M., and Lanyi, J. K. (1984) *J. Biol. Chem.* 259, 2179-2184), this site was not detected by ^{35}Cl NMR, suggesting that the transport site is in the interior of the protein where it is sampled slowly by chloride in the medium. It is proposed that the purified chromoprotein possesses a channel leading from the medium to the transport site and that the channel contains the high affinity NMR site which facilitates the migration of chloride between the medium and the transport site.

We have also used ^{35}Cl NMR to study chloride binding to purified monomeric bacteriorhodopsin; however, this protein contains no detectable chloride-binding sites.

presence of light and extracellular chloride, the chromophore undergoes a photocycle that is accompanied by transport of chloride into the intracellular space (2, 3). An apparent $M_r = 20,000$ polypeptide containing the chromophore has recently been isolated (4-6). This purified chromoprotein exhibits a chloride-dependent photocycle that is virtually indistinguishable from that of native halorhodopsin (7); thus, the chromoprotein probably contains the chloride-dependent part of the halorhodopsin transport unit.

The mechanism of chloride transport by halorhodopsin is not yet known. The simplest possible mechanism for this process would involve a transport site that binds substrate chloride ion and carries it through part or all of the transport cycle. Evidence that such a site exists has already been obtained in transport studies (1, 3) and in optical studies of the effect of chloride binding on the chromophore (3, 7). We have recently used a ^{35}Cl NMR assay to study chloride binding to the transport sites of the band 3 protein of human red cells. Unlike halorhodopsin, band 3 is a passive transport protein; like halorhodopsin, however, band 3 translocates chloride across a cell membrane. In the band 3 system, ^{35}Cl NMR enables stringent tests of the mechanism of anion transport,^{1,2} and in principle similar mechanistic information could be obtained in the halorhodopsin system. In the following, we describe a ^{35}Cl NMR study of chloride binding to the purified halorhodopsin chromoprotein. The ^{35}Cl NMR assay reveals two previously unknown types of chloride-binding sites on the chromoprotein: a low affinity class of sites which might be nonspecific sites, and a high affinity class which might facilitate migration of chloride through a channel leading to the transport sites. In addition, we show that the halorhodopsin transport sites are not detected by the assay, suggesting that these sites are in the interior of the protein so that access of solution chloride to the sites is slow.

MATERIALS AND METHODS

Preparations of purified halorhodopsin chromoprotein (6) and of purified monomeric bacteriorhodopsin were carried out exactly as described in the preceding paper (7). These preparations yielded purified protein in 1 M NaCl, 10 mM MOPS,³ 1% octyl glucoside, pH 7.0. For NMR samples, the chloride concentration was lowered to 100 mM NaCl or to <1 mM NaCl as appropriate by dialysis against 0.5 M Na_2SO_4 , 10 mM MOPS, pH 7.0 \pm 100 mM NaCl as described in

Halorhodopsin is a light-driven chloride pump in the cell membrane of *Halobacterium halobium* (1). This protein has a chromophoric site containing the pigment retinal, and in the

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³ The abbreviation used is: MOPS, 3-(N-morpholino)propane-sulfonic acid.

the preceding paper (7). Because of the instability of the halorhodopsin chromoprotein at low chloride concentrations (7), care was taken to keep the chloride-free chromoprotein samples on ice in the dark and to use the samples within 3 h of completion of dialysis.

All NMR samples were prepared using the same general procedure. Purified protein (typically 40 nmol) was diluted to 4 ml with the appropriate buffer to yield protein in NMR buffer (0.5 M Na₂SO₄, 10 mM MOPS, pH 7.0 with NaOH, 10% D₂O, \pm 100 mM NaCl). Titrations with chloride were performed on a single sample, initially chloride-free, by successive additions of 4 M NaCl, followed by acquisition of a ³⁵Cl NMR spectrum for each new chloride concentration. Titrations with anions other than chloride were on a single sample for each anion X⁻, containing 100 mM NaCl, by successive additions of 4 M NaX + 100 mM NaCl, followed by acquisition of a ³⁵Cl NMR spectrum at each new anion concentration. Exceptions were fluoride and citrate, which were added as the stock solutions 1 M NaF, 100 mM NaCl or 0.67 M citrate, 100 mM NaCl. Titrations of pH were on a single sample containing 100 mM NaCl by successive additions of 0.2 N NaOH or 0.1 N H₂SO₄, followed by acquisition of a ³⁵Cl NMR spectrum at each new pH. Where indicated, the high affinity sites were isolated by the addition of 4 M NaBr or 4 M NaBr + 100 mM NaCl to yield 800 mM NaBr \pm 100 mM NaCl. All NaX stock solutions were titrated to pH 7.0 with NaOH or H₂SO₄.

In one experiment, a chloride-free sample of halorhodopsin chromoprotein was bleached by illumination for 5 min on ice with the focused beam of a 200-watt mercury-xenon lamp, using a 530-nm longpass filter. Following bleaching, the sample was titrated with chloride as above, except before each spectrum the sample was again illuminated for 2 min to ensure the irreversibility of the bleaching.

³⁵Cl NMR spectroscopy was conducted as described elsewhere¹ at 10 °C using a Varian XL-200 NMR spectrometer (³⁵Cl resonance frequency 19.6 MHz). Each 10-mm diameter sample tube contained \geq 4 ml of sample. Before acquisition was started, 1,000 nonacquired scans were taken to ensure equilibration of the sample temperature. Then, 1,000–20,000 scans were acquired, the resulting solution chloride ³⁵Cl NMR spectrum was recorded, and the line width at half-height of the spectrum was measured with a ruler. The ³⁵Cl⁻ line broadening of a sample was calculated as the difference between the line width of that sample and the line width of a sample containing buffer but no protein. Titrations with chloride and other anions resulted in significant increases in the total sample volume. These volume increases were recorded and were considered when calculating anion concentrations. The volume increases also caused dilution of the protein, thereby necessitating correction of the observed ³⁵Cl⁻ line broadening by the multiplication factor (initial protein concentration)/(diluted protein concentration), see Equation 2 in text.

RESULTS

The ³⁵Cl NMR Assay for Chloride Binding to Halorhodopsin

The binding of chloride to halorhodopsin can be studied using a ³⁵Cl NMR binding assay that has been widely applied in studies of anion-binding sites on proteins (8). The physical basis of this assay is the large difference in the spectral widths of bound and free chloride; the spectral width of chloride bound to a macromolecule is typically $\geq 10^4$ times larger than the spectral width of solution chloride. Because of the large spectral width and relatively small concentration of bound chloride (in our experiments [total protein]/[total chloride] $\leq 10^{-3}$), the observed ³⁵Cl NMR resonance is essentially that of solution chloride alone. However, when chloride exchanges sufficiently rapidly between binding sites and solution, the sites can cause a measurable increase in the line width of the observed resonance (8).¹ For a population of identical protein molecules that each possess *only one type* of site, the increase in line width (or line broadening) is given by¹

$$\delta_i = [P] \cdot \frac{\alpha_i \cdot n_i}{K_{D_i}} \cdot \frac{[Cl^-]^{-1}}{[Cl^-]^{-1} + K_{D_i}^{-1}} \quad (1)$$

where [P] is the protein concentration, α_i is a proportionality constant characteristic of the *i*th type of site, K_{D_i} is the chloride dissociation constant of the *i*th type of site, n_i is the number of such sites on a single molecule of protein, and [Cl⁻]

is the total chloride concentration. For a population of identical protein molecules that each possess *multiple types* of sites, the line broadening due to the different types of sites is *additive* and the observed line broadening becomes¹

$$\delta = \sum_i \delta_i = [P] \cdot \sum_i \frac{\alpha_i \cdot n_i}{K_{D_i}} \cdot \frac{[Cl^-]^{-1}}{[Cl^-]^{-1} + K_{D_i}^{-1}} \quad (2)$$

Both Equations 1 and 2 assume that (a) the concentration of bound chloride is negligible relative to the concentration of free chloride and (b) the bound chloride returns to solution before binding to another site.

The usefulness of the ³⁵Cl NMR assay stems from the linear relationship between the line broadening and the concentration of the *i*th type of site ($= [P] \cdot n_i$). This linear relationship makes the line broadening directly proportional to the protein concentration (Equations 1 and 2), a property that is experimentally verified in a plot of line broadening *versus* the concentration of purified halorhodopsin chromoprotein (Fig. 1). These results indicate that the chromoprotein contains one or more types of chloride-binding sites. The number of the observed sites/chromoprotein molecule (n_i ; Equations 1 and 2) cannot be determined at the present time because the constants α_i are unknown in the halorhodopsin system; however, other characteristics of the observed sites can still be determined using the ³⁵Cl NMR assay.

Characteristics of the Observed Sites

Chloride Affinity—The chloride affinity of the observed chromoprotein sites can be determined from their line broadening. According to Equation 1, a plot of line broadening *versus* [Cl⁻]⁻¹ can be used to operationally define a site as a high affinity or a low affinity site; a high affinity site ($K_{D_i} \leq [Cl^-]$) gives rise to a square hyperbola on such a plot, while a low affinity site ($K_{D_i} \gg [Cl^-]$) gives rise to a straight line of zero slope. For the purified halorhodopsin chromoprotein, a line broadening *versus* [Cl⁻]⁻¹ plot (Fig. 2) reveals a class of identical high affinity sites ($K_{D_i} = 110 \pm 50$ mM) plus a second class of low affinity sites ($K_{D_i} \gg 1$ M). Both types of sites have K_{D_i} values that are significantly larger than the values measured in transport studies (1, 3) and in optical studies on the effect of chloride on the halorhodopsin chromophore (3,

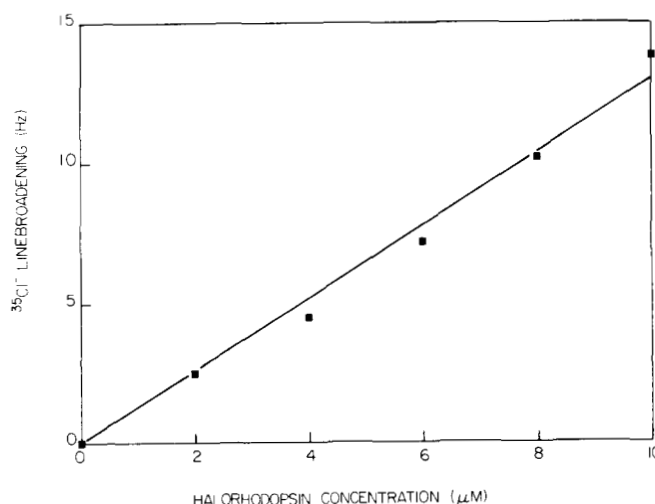


FIG. 1. Effect of halorhodopsin on the ³⁵Cl⁻ line broadening. The ³⁵Cl⁻ line broadening due to the purified halorhodopsin chromoprotein in NMR buffer ([Cl⁻] = 100 mM) was measured as described in the text. The 10 μM sample was measured first; successive dilution yielded the subsequent samples. Results were fit by linear least squares (solid line).

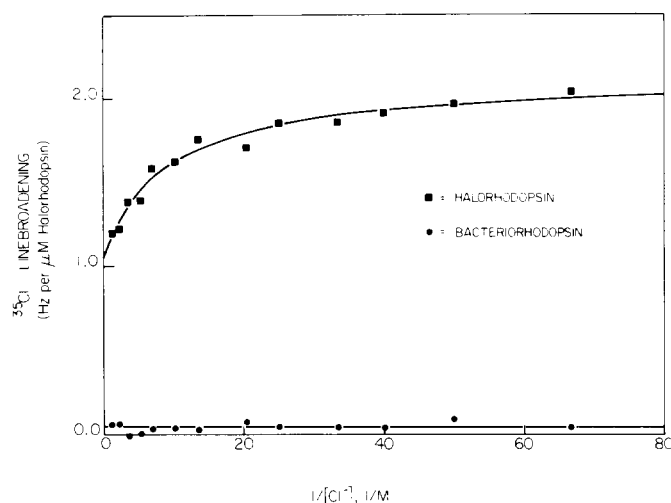


FIG. 2. The $^{35}\text{Cl}^-$ line broadening due to halorhodopsin and bacteriorhodopsin. The $^{35}\text{Cl}^-$ line broadening due to purified protein in NMR buffer was measured as described in the text. Results were best fit by linear least squares (lower curve, $K_D \gg 1\text{ M}$) or by nonlinear least squares (upper curve, best fit for a population containing both high affinity sites ($K_D = 110 \pm 20\text{ mM}$) and low affinity sites ($K_D \gg 1\text{ M}$)).

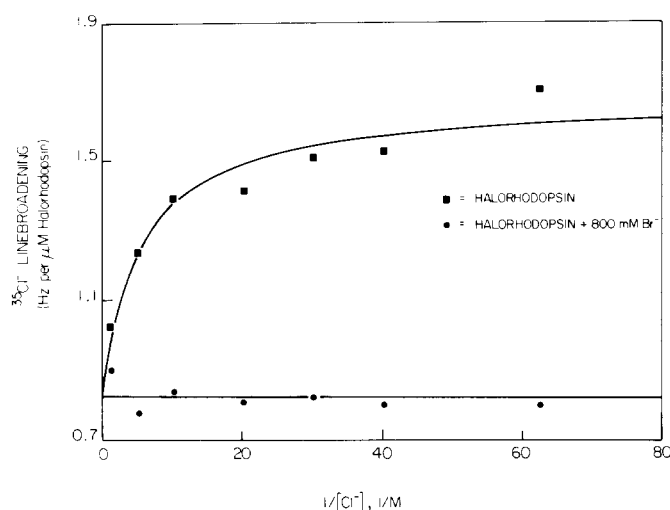


FIG. 3. Inhibition of the high affinity NMR sites by Br^- . The $^{35}\text{Cl}^-$ line broadening due to the purified halorhodopsin chromoprotein in NMR buffer was measured as described in the text. Results were fit by linear least squares (lower curve, $K_D \gg 1\text{ M}$) or by nonlinear least squares (upper curve, best fit for a population containing both high affinity sites ($K_D = 180 \pm 30\text{ mM}$) and low affinity sites ($K_D \gg 1\text{ M}$)).

7). Thus, the sites revealed by ^{35}Cl NMR are new sites that have not been previously observed. These new sites will collectively be termed the NMR sites.

Affinity for Other Anions—The NMR sites can be characterized with respect to the binding of any anion, as long as that anion competes with chloride for binding. Such an anion inhibits the observed line broadening by reducing the effective number of chloride-binding sites (n_i ; Equation 2). For the purified halorhodopsin chromoprotein, the line broadening due to the high affinity sites is eliminated by 800 mM bromide (Fig. 3); these results indicate that 800 mM bromide saturates the sites and thereby reduces n_i to zero. In contrast, the line broadening due to the low affinity sites is unaffected (Fig. 3); thus, even in the presence of 800 mM bromide, the low affinity sites are far from saturation.

Since bromide is a competitive inhibitor of the high affinity sites, the bromide affinity of these sites can be determined by varying the bromide concentration. When the concentration of the competitive inhibitor X is varied while keeping the chromoprotein and chloride concentrations fixed, the line broadening is given by ¹

$$\delta = \delta_H(0) \cdot \left(1 - \frac{[X]}{[X] + K_I(\text{app})}\right) + \delta_L \quad (3)$$

where $\delta_H(0)$ is the line broadening of the high affinity sites in the absence of inhibitor, δ_L is the line broadening of the low affinity sites, and $K_I(\text{app})$ is the apparent dissociation constant for inhibitor binding in the presence of the fixed chloride concentration. For the purified halorhodopsin chromoprotein, a plot of line broadening versus $[\text{Br}^-]$ in the presence of 100 mM chloride yields an apparent dissociation constant of $66 \pm 5\text{ mM}$ for bromide (Fig. 4). Other anions that compete with chloride for binding to the high affinity sites have also been studied, as summarized in Table I. The apparent dissociation constants of these anions can be corrected

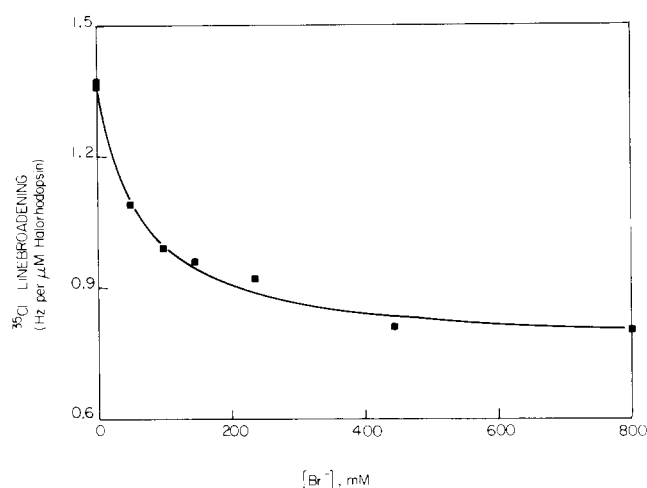


FIG. 4. Effect of varying $[\text{Br}^-]$ on the $^{35}\text{Cl}^-$ line broadening due to halorhodopsin. The $^{35}\text{Cl}^-$ line broadening due to the purified halorhodopsin chromoprotein in NMR buffer ($[\text{Cl}^-] = 100\text{ mM}$) was measured as described in the text. Results were fit by nonlinear least squares (best fit $K_D = 66 \pm 5\text{ mM}$) using Equation 3 in text.

TABLE I

Dissociation constants for anion binding to the high affinity NMR sites

Anion (mM)	Interaction with the high affinity NMR sites ^a		
	Fractional inhibition ^b	Apparent ^c K_D mM	Corrected ^d K_D mM
Cl^-			110 ± 50^e
I^- (50)	0.93	4	2
SCN^- (50)	0.87	7	4
Br^- (50)	0.48	53 (66 ± 5) ^f	26 (33 ± 5) ^f
NO_3^- (50)	0.32	110	55
F^- (50)	0.04	>1000	>500
Citrate (170)	0.00	>1000	>500

^a The $^{35}\text{Cl}^-$ line broadening of the high affinity sites was the line broadening inhibited by 800 mM Br^- .

^b Fractional reduction of the high affinity site line broadening caused by anion X^- .

^c Calculated from Equation 3 in text ($[\text{Cl}^-] = 100\text{ mM}$).

^d Calculated from Equation 4 in text, assuming X^- competitively inhibits Cl^- binding to the high affinity site.

^e From Fig. 2 and three similar experiments.

^f From Fig. 4.

for the presence of chloride using the relationship¹

$$K_I(\text{app}) = K_I \left(1 + \frac{[\text{Cl}^-]}{K_{D_i}} \right) \quad (4)$$

where K_I is the true dissociation constant for the competing anion and K_{D_i} is the dissociation constant for chloride binding ($=110 \pm 50$ mM). The cumulative results (Table I) indicate that the relative affinities of the high affinity site for the anions studied are $\text{I}^- > \text{SCN}^- > \text{Br}^- > \text{NO}_3^- > \text{Cl}^- > \text{F}^-$, citrate. This affinity series is significantly different than that observed for other anion-binding sites on the chromoprotein (7), thereby emphasizing the uniqueness of the NMR sites.

The above analysis assumes that chloride and the inhibitory anions bind to the chromoprotein in a mutually exclusive fashion. Yet a different explanation for line broadening inhibition by the anions can also be proposed; the non-chloride anions could bind to a second site separate from the high affinity NMR sites, thereby slowing the exchange of chloride between the intact high affinity NMR sites and solution. The second site must not bind chloride since no line broadening inhibition is observed at large chloride concentration (Fig. 2). However, the second site would have to bind a variety of anions other than chloride (Table I), including the halides bromide and iodide. It is unlikely that a site which is otherwise relatively nonspecific would exclude chloride binding; thus, the data support the conclusion that the inhibitory anions are competitive inhibitors of chloride binding to the high affinity NMR sites.

pH Dependence

Information concerning the structure of the NMR sites can be obtained from the pH dependence of the line broadening due to those sites. In general, anion-binding sites contain at least one essential positive charge provided by protonated groups. When an essential charge is titrated away by base, chloride will no longer bind and the number of sites/protein molecule (n_i ; Equation 2) is decreased, thereby causing line broadening inhibition. Measurement of line broadening requires the presence of a fixed concentration of chloride, which could bind to and stabilize the essential charge, and thereby artifactually increase the pK_a . However, in the case at hand ($[\text{Cl}^-] = 100$ mM; $K_D = 110$ mM), the increase is <0.3 pH units and is thus a minor effect (9).

Titration of the NMR sites of the purified halorhodopsin chromoprotein with NaOH yields the data of Fig. 5. The line broadening of the high affinity sites is unaffected as the pH varies from pH 7 to 11, but between pH 11 and 12 a decrease is observed that is not fully reversed by back titration with H_2SO_4 . This irreversible line broadening decrease is probably the result of denaturation, not simple deprotonation. Thus, at pH 11, most of the high affinity sites remain protonated. This indicates that arginine, rather than lysine or the protonated Schiff's base of the chromophore, provides the positive charge in these sites. Similarly, most of the low affinity sites remain protonated at pH 12 (Fig. 5); thus, arginine also provides the positive charge in these sites.

Effect of Bleaching

If the NMR sites are closely associated with the halorhodopsin chromophore, then bleaching of the chromophore could measurably affect the line broadening due to the sites. Here bleaching of the chromophore was carried out in two ways: (a) by exposure to high pH in the presence of chloride (Fig. 5) and (b) by intense illumination at neutral pH in the absence of chloride. Both types of bleaching appear to cause

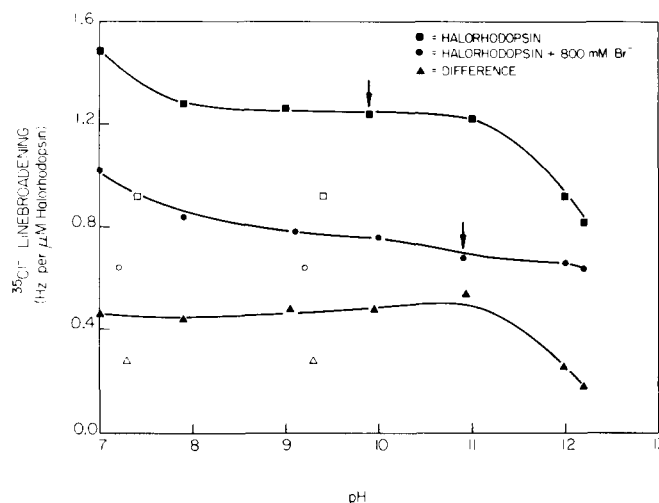


FIG. 5. pH dependence of the $^{35}\text{Cl}^-$ line broadening due to halorhodopsin. The $^{35}\text{Cl}^-$ line broadening due to the purified halorhodopsin chromoprotein in NMR buffer ($[\text{Cl}^-] = 100$ mM) was measured, starting at pH 7 and increasing to pH 12.2 using NaOH. Bleaching of chromophore occurred spontaneously in room light at arrow. Samples were back-titrated using H_2SO_4 (open symbols).

deprotonation of the protonated Schiff's base, thereby shifting the chromophore absorption maximum from 580 nm to near 400 nm and producing a noticeable color change (6, 10). Here the color change was not reversible during the time course of the experiment (>30 min); thus, in addition to deprotonation, the bleaching must cause significant structural changes in the chromophore and/or in the protein structure proximal to the chromophore. Yet the first type of bleaching does not affect the line broadening due to the purified halorhodopsin chromoprotein (Fig. 5). Similarly, the second type of bleaching affects neither the line broadening nor the chloride affinity of the high affinity ($K_D = 135 \pm 50$ mM after bleaching) and low affinity ($K_D \gg 1$ M after bleaching) NMR sites (not shown). These results are consistent with a picture in which both kinds of NMR sites are distant from the chromophore and are not involved in the transport step of the halorhodopsin photocycle.

Bacteriorhodopsin

Purified monomeric bacteriorhodopsin has also been studied using the ^{35}Cl NMR assay; however, the line broadening due to this protein is negligible (Fig. 2). It should be mentioned, however, that a low affinity binding site ($K_D \approx 1$ M) for chloride and other anions was found in bacteriorhodopsin in purple membranes at very acidic pH values (pH 1). This binding site was interpreted as the replacement of the intrinsic negative counterion of the protein's Schiff's base by an external anion (11). Nevertheless, together with the ^{35}Cl NMR evidence from the halorhodopsin system, these results indicate that halorhodopsin is specialized to bind chloride, while bacteriorhodopsin is not.

DISCUSSION

Several types of chloride-binding sites are now known to exist in the halorhodopsin system (this and the preceding paper (7)). The existence of transport sites is suggested by the saturation of chloride transport that occurs in vesicles at sufficiently large chloride concentrations (1).⁴ This saturation

⁴ E. Bamberg, P. Hegemann, and D. Oesterhelt, submitted to *Proc. Natl. Acad. Sci. U. S. A.*

behavior exhibits a half-saturation constant that agrees well with the dissociation constant for chloride binding to a site that affects the chromophore in optical experiments (3, 10); therefore, this "optical" site is probably the transport site. The preceding paper (7) shows that the optical site is associated with the purified halorhodopsin chromoprotein, and in this purified system, the site exhibits a chloride dissociation constant of $K_D \cong 10$ mM (7). The preceding paper also raises the possibility that a second optical site may exist that is distinct from the transport optical site. Neither of these optical sites are observed in the present study, which employs ^{35}Cl NMR to monitor chloride binding to a preparation of purified chromoprotein identical to that used in the preceding paper. The fact that the optical sites, including the proposed transport site, are not detected by the ^{35}Cl NMR assay indicates that for the optical sites the quantity α_i (Equation 2) tends to zero. This condition occurs when the symmetry of the binding site is tetrahedral or higher or when the exchange of chloride between the site and solution is too slow.¹

Although the optical sites are not detected, two new classes of sites on the purified chromoprotein are revealed by the ^{35}Cl NMR assay; these NMR sites have chloride dissociation constants of 110 ± 50 mM and $>>1$ M. Both types of NMR sites (a) are distinguished from the optical sites by a variety of criteria, (b) appear to contain arginine as the essential positive charge, and (c) are unaffected by bleaching of the halorhodopsin chromophore. The insensitivity of the NMR sites to bleaching suggests that they are not in the immediate vicinity of the chromophore and are not directly involved in the transport step of the halorhodopsin photocycle.

All of the ^{35}Cl NMR results are consistent with the following model. The halorhodopsin transport unit contains a transport site and possibly a second optical site which exist in the interior of the protein in the vicinity of the chromophore. A channel leads from these sites to the extracellular solution, which is the source of substrate chloride. The high affinity

NMR site is found in this channel, and the function of this site is to facilitate the diffusion of chloride through the channel. The low affinity NMR sites are nonspecific chloride-binding sites on the surface of the protein. However, these low-affinity sites may be responsible for the transport inhibition observed at $[\text{Cl}^-] > 1$ M (3); a similar inhibitory role has been proposed for low affinity sites in the band 3 anion-transport system.¹

The ^{35}Cl NMR results also emphasize the functional difference between bacteriorhodopsin and halorhodopsin. At pH 7, bacteriorhodopsin appears to lack completely chloride-binding sites, in contrast to halorhodopsin. This evidence supports the current picture in which bacteriorhodopsin is specialized to transport protons, while halorhodopsin is specialized to transport chloride.

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